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Introduction

β -catenin is an oncogene involved in signaling by the wnt pathway. Upon wnt signaling, β -catenin accumulates in the cytoplasm and nucleus where it interacts with Tcf/LEF transcription factors. This complex then activates transcription of several genes, including cyclin D1 and c-myc, indicating that this pathway may be involved in cell cycle regulation (12;19;30;37;41). In the absence of wnt signaling, cytoplasmic β -catenin associates with a complex containing the tumor suppressor gene adenomatous polyposis coli (APC), axin, and GSK3 β (27-29;32). The activity of this complex results in phosphorylation of β -catenin at N-terminal serine and threonine residues. Mutations in this N-terminal region or mutations of the APC gene lead to accumulation of β -catenin and are associated with many different cancers, including melanoma and colon cancer (reviewed in (26)). Once phosphorylated β -catenin is recognized by F box/WD 40 repeat containing proteins, targeting it for ubiquitination by the SCF E3 ubiquitin ligase and subsequent proteasomal degradation (8;11;18;43). Although GSK-3 β is clearly involved in the wnt-mediated regulation of β -catenin stability, several studies indicate that other kinases may also be involved in regulation of β -catenin signaling. For example, both typical and atypical PKCs have been implicated in β -catenin regulation (5;29). However, LiCl, an inhibitor of GSK3 β , does not significantly alter the ability of APC to down-regulate β -catenin activity (7). In addition, the GSK3 β knockout mouse does not exhibit any changes in β -catenin levels and is morphologically normal up to 12 days of gestation(13). Remarkably, GSK3 β knockout mice are characterized by defects similar to those of mice lacking components of the NF κ B pathway.

There are several other connections between β -catenin regulation and regulation of I κ B α , which inhibits NF- κ B by sequestering it in the cytoplasm. The I κ B α kinase complex, IKK, regulates NF- κ B activation by phosphorylating its inhibitors I κ B α , I κ B β , and I κ B ϵ on two closely spaced serine residues and targeting them for ubiquitination using the same F box/SCF machinery used for ubiquitination of β -catenin (8;20;25;33;43). IKK recognizes and phosphorylates I κ B α at a DSGIHS consensus sequence in the N-terminal region including serines 32 and 36. This consensus sequence is also found in the N-terminal of β -catenin at serines 33 and 37 (29).

The IKK complex is composed of a number of different proteins including the active catalytic components IKK α and IKK β and the scaffold protein IKK γ /NEMO (34;44) (15). Several different stimuli including tumor necrosis factor α (TNF α) and interleukin 1 (IL-1) can stimulate the activity of the IKK complex. A number of kinases including MEKK1, NF κ B inducing kinase (NIK), and atypical PKCs can activate IKK (17;22;26;27). Mechanical stress acting through integrins can also activate IKK (2). Once activated, the IKK complex phosphorylates I κ B proteins, which lead to their degradation resulting in the release of the NF- κ B proteins and their migration to the nucleus where they activate transcription of certain cellular genes. The NF- κ B proteins are known to play a role in inflammatory responses as well as in apoptosis, cell survival, and development (reviewed in (17)).

IKK β has higher activity for the I κ B proteins and has a more significant role in the NF- κ B pathway in response to activation such as TNF α and IL-1 than does IKK α (6;14;21;22;24;40). For example, IKK β knockout mice die around embryonic day 12.5 from severe liver apoptosis, a phenotype that is similar to knockouts of the NF- κ B component p65 and GSK3 β . Fibroblasts taken from these embryos demonstrate decreased NF- κ B activity and increased apoptosis due to unopposed TNF α stimulation. IKK α cannot compensate for the loss of IKK β indicating that IKK β is absolutely necessary for the regulation of this pathway. Knockouts of the IKK γ /NEMO gene which is critical for the control of IKK β activity also result in severe hepatic apoptosis and defects in NF- κ B activation in response to TNF α .

and IL-1 treatment (35). In contrast, IKK α knockouts die perinatally with skeletal abnormalities including fused vertebrae, syndactyly, and missing phalanges. Upon TNF α and IL-1 stimulation, IKK α -/- cells exhibit somewhat reduced I κ B α degradation and NF κ B activity but the defect is not as severe as seen with disruption of the IKK β gene. Moreover, these mice do not exhibit the severe hepatic apoptosis that characterizes the IKK β , IKK γ /NEMO, and GSK-3 β knockout mice. These results demonstrate different roles for IKK α and IKK β . In addition, IKK α knockout mice exhibit an unusual 10-fold thickening of the epidermis due to hyperproliferation of basal keratinocyte stem cells. Significantly, increased β -catenin levels are also associated with uncontrolled proliferation of epidermal basal cells and there are examples of wnt and LEF-1 involvement in hair and epidermal differentiation (10;45).

In the past 12 months we investigated the ability of IKK to regulate β -catenin signaling activity. We found that IKK exists in a complex with β -catenin and that expression of either IKK α or IKK β can decrease β -catenin signaling. However, only a dominant negative (DN) IKK α mutant increased β -catenin signaling as well as protein levels. In addition, DN IKK α , but not DN IKK β , inhibited the ability of APC to decrease β -catenin signaling. These results suggest that the IKK proteins are involved in β -catenin regulation.

Materials and Methods:

Cell culture and Transfections: SW480 colon cancer cells, SKBR3 breast cancer cells, and Cos-7 kidney cells were grown in DMEM supplemented with 5% fetal bovine serum. SW480 cells were transfected using lipofectamine plus (GibcoBRL). SKBR3 and Cos-7 cells were transfected using calcium phosphate (Promega). F. Mercurio kindly provided the IKK α (S176/180E and A) and IKK β (S177/181E and A) mutants (25). B. Vogelstein kindly provided APC. R. Pestell kindly provided the cyclin D1-luciferase promoter. Tularik Inc kindly provided the IKK α (K44M) and IKK β (K44A) (ref?).

Tcf/LEF Reporter Assay: Cells were plated at ~100,000 cells/well in a 12 well plate. After 24 hours, cells were transfected with the indicated DNA as well as TopFlash, which is a luciferase reporter containing Tcf/LEF response elements, or FopFlash, which is a Tcf/LEF mutated luciferase reporter, (described in (42)) and Renilla luciferase. All transfections were done in triplicate and repeated at least three times with the Tcf/LEF reporter activity measured in lumens after 48 hours using the luciferase assay (Promega). Dose responses were also performed and optimal doses chosen for these experiments.

Western Blotting: Cells were grown to confluence in 100 mm dishes (48 hours after transfection), washed twice with phosphate buffered saline (PBS), and lysed for 10 minutes on ice with NP-40 lysis buffer containing 1% NP-40 and protease inhibitors (1mM sodium vanadate, 50 mM sodium fluoride, and Boehringer Mannheim complete mini EDTA-free protease inhibitor cocktail). Lysates were centrifuged at 12,000 rpm at 4°C for 10 minutes. Protein content was measured by the BCA protein assay (Pierce). Western blotting was performed as previously described using β -catenin monoclonal antibody (Transduction Laboratories) at a 1:1000 dilution in 5% milk (38). Monoclonal HA antibody (Boehringer Mannheim) was used at a concentration of 1 μ g/ml, monoclonal FLAG antibody (Eastman Kodak) was used at a 1:500 dilution, and myc antibody was used at a concentration of 1 μ g/ml. The blots were developed using chemiluminescent detection (Pierce).

Immunocytochemistry: Cells were plated on 18mm coverslips in 12 well plates at ~ 50,000 cells/well. Cells were transfected as above and fixed 48 hours later in 2% paraformaldehyde and permeabilized with 0.2% Triton. Monoclonal HA antibody (Babco) was used at a 1:1000 dilution and monoclonal FLAG antibody was used at a 1:500 dilution. Polyclonal β -catenin antibody (kindly provided by David Rimm)

was used at a 1:2000 dilution. All primary antibodies were incubated for 1 hour at room temperature. Fluorescein and Texas Red conjugated secondary antibodies (Kirkegaard and Perry Laboratories) were used at a 1:100 dilution and incubated for 1 hour at room temperature. Imaging was done on an Olympus Fluoview Confocal Laser Scanning Microscope.

Fractionation of Cellular Extracts: Cytoplasmic extracts were prepared according to Li et al (23) with slight modifications. Cos-7 cells were plated in 100 mM dishes (10^7 cells) and co-transfected with Flag-tagged IKK α and HA-tagged β -catenin. Prior to harvest, cells were washed twice with cold phosphate-buffered saline (PBS). Cells were next resuspended in buffer A (10 mM Hepes [pH 7.9], 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM EDTA) supplemented with phosphatase inhibitors (10 mM NaF, 10 mM β -glycerolphosphate, 0.5 μ M okadaic acid, 1 mM sodium orthovanadate) and protease inhibitors (Roche Molecular Biochemicals). After incubation on ice for 10 min, cells were lysed in Wheaton all-glass Dounce homogenizer (Tight pestle). Nuclei were pelleted by centrifugation for 5 min at 2000 rpm (Beckman bench-top centrifuge, CH3.7 rotor). The supernatants were collected and mixed with 0.11 volume of buffer B (0.3 M Hepes [pH 7.9], 30 mM MgCl₂, and 1.4 M NaCl) and then centrifuged at 100,000 xg for 60 min at 4°C. The supernatants after ultra-centrifugation were termed S100.

Gel Filtration Chromatography: S100 were further fractionated on a Superdex-200 gel filtration column (Amersham Pharmacia Biotech) equilibrated with buffer D (20 mM Hepes [pH 7.9], 0.1 M KCl, 0.5 mM PMSF, 0.5 mM dithiothreitol, 0.2 mM EDTA, 20% glycerol). Protein markers (Sigma) used for the calibration of the column include bovine thyroglobulin (669 kDa), horse spleen apoferritin (443 kDa), α -amylase (200 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.5 kDa).

Protein Association Studies and Western Blot Analysis: For protein association studies, equal volumes from each of the Superdex-200 fraction were incubated overnight at 4 °C with 1 μ g of indicated antibodies or normal mouse IgG. Immune complexes were collected with protein G-agarose (Sigma) for 2-3 h at 4 °C. Immunoprecipitates were analyzed by Western blotting using a chemiluminescence system (ECL-Amersham Pharmacia Biotech).

Results:

To determine whether IKK could regulate β -catenin signaling we first transfected SW480 colon cancer cells with the constitutively active IKK mutants and a reporter construct, which reflects β -catenin activity. These constitutively active mutants (CA IKK α and CA IKK β (SS EE)) were generated by changing serine residues located within the highly conserved activation loop at position 176 and 180 in IKK α and 177 and 181 in IKK β , which mimic the phosphorylation induced conformational change resulting in kinase activation. SW480 cells contain a truncated APC gene and as a result have very high β -catenin levels and signaling. To measure β -catenin signaling, we used a luciferase promoter containing Tcf/LEF response elements (TopFlash) and measured the lumens generated. A Tcf/LEF mutated luciferase reporter (FopFlash) was used as a control. β -catenin signaling decreased by 80-90% upon co-transfection of either CA IKK α or CA IKK β expression, (Figure 1A). In contrast, CA IKK α increased NF- κ B reporter activity in the same experiment. To further investigate the requirement for IKK kinase activity, we next transfected kinase dead IKK α and β mutants, in which a conserved lysine in the ATP binding site at position 44 was mutated to either methionine in IKK α or alanine in IKK β , into SW480 cells. Neither the kinase dead IKK α nor IKK β mutant decreased β -catenin activity indicating that intact

kinase function for both IKK α and β is necessary for their effects on β -catenin signaling (Figure 1B). These data indicate that IKK α and IKK β are capable of regulating β -catenin signaling.

To more clearly address the role of these kinases, we transfected either dominant negative (DN) IKK α (S176/180A) or IKK β (S177/181A) into SKBR3 breast cancer cells (Figure 1C). The alanine substitutions for serine residues in the activation loops of these kinases prevent kinase activation in response to TNF α and IL-1 stimulation. SKBR3 cells have very low levels of β -catenin and consequently the low levels of β -catenin activity permit easier detection of changes in its signaling activity. The DN IKK α increased β -catenin signaling over 5 fold and significantly increased β -catenin protein levels (Figure 1C). In contrast, the DN IKK β had no effect on β -catenin signaling or protein levels. Neither CA IKK α nor CA IKK β affected β -catenin signaling or protein levels in SKBR3 cells. These results indicate that blockade of endogenous IKK α activation but not of IKK β activation has a direct effect on the activity of the IKK complex toward β -catenin in SKBR3 cells. This result contrasts with failure of DN IKK α to block IKK activity toward I κ B α . We next investigated the ability of DN IKK α to regulate the phosphorylation-dependent molecular weight shift observed in SKBR3 cells when β -catenin is over-expressed (Figure 1D). Consistent with a role for IKK α in regulating β -catenin phosphorylation the higher molecular weight form of β -catenin was absent in cells co-expressing DN IKK α . Western blot analysis was then performed to determine the levels of endogenous IKK α and β in the different cell lines used in these studies. We found SKBR3, SW480, and Cos-7 cells to have significantly more IKK α than IKK β using the indicated IKK α and IKK β antibodies while Jurkat cells, which were used as a positive control, expressed similar levels of IKK α and β (Figure 1E).

To determine whether IKK α influences the cellular localization of β -catenin, immunocytochemistry was performed using SKBR3 cells transfected with either HA-tagged DN IKK α or FLAG-tagged DN IKK β . Cells were stained for either IKK α or IKK β using antibodies directed against either the HA or FLAG tag as well as β -catenin. DN IKK α was found in both the nucleus and the cytoplasm in the majority of transfected cells (Figure 2 A1). β -catenin levels were significantly increased in DN IKK α transfected cells and in most cases β -catenin co-localized with DN IKK α in the nucleus (Figure 2 A2). In cells transfected with DN IKK β , IKK β was found only in the cytoplasm (Figure 2 B1) and there was no change in β -catenin levels or localization (Figure 2 B2). CA IKK α also localized to the nucleus (not shown).

To demonstrate whether IKK could regulate β -catenin activated transcription of a known target gene, a cyclin D1-luciferase promoter was transfected into SW480 cells. This cyclin D1 promoter construct (-163CD1Luc) contains Tcf/LEF sites as well as CREB, AP1, SP1, and NF- κ B sites (31;37). As was the case with the TCF-reporter, TopFlash, both the constitutively active IKK α and IKK β proteins decreased cyclin D1 promoter activity in SW480 cells (Figure 3). In contrast, a cyclin D1 promoter with mutated Tcf/LEF sites, but retaining the other regulatory elements, was not responsive to either IKK α or IKK β . Because NF κ B has been shown to positively regulate cyclin D1 promoter activity these data indicate that, in APC-mutant colon cancer cells, the activity of the -163 cyclin D1 promoter is predominantly regulated by IKK control of β -catenin not NF- κ B signaling.

When wild type (WT) APC is over-expressed in SW480 cells, β -catenin signaling is dramatically reduced. In earlier studies, we found that GSK3 β activity was not required for APC to decrease β -catenin signaling (7). To determine if IKK activity was required for APC to regulate β -catenin activity, SW480 cells were co-transfected with APC and DN IKK α or DN IKK β (Figure 4). Remarkably, DN IKK α but

not DN IKK β completely inhibited the ability of APC to decrease β -catenin signaling. These results suggest that IKK α can directly alter β -catenin signaling perhaps by association with the IKK complex.

Finally, to determine whether IKK α could directly interact with β -catenin, immunoprecipitation was performed in Cos-7 kidney cells co-transfected with HA-tagged β -catenin and FLAG-tagged IKK α . Before immunoprecipitation cytoplasmic cell extracts were fractionated over a sizing column. Figure 5 shows that β -catenin is present in IKK α immunoprecipitates and that IKK α is also present in β -catenin immunoprecipitates. Thus IKK α can directly associate with a protein complex, which includes β -catenin.

Several studies point to separate roles for IKK α and IKK β in regulating the activity of the IKK complex (reviewed in (16)). IKK β activity is essential for the ability of the IKK complex to regulate I κ B protein stability and thus control the transcription of NF- κ B regulated genes. Although both exogenously expressed and endogenous IKK α can regulate NF- κ B activity, it does not appear to have a dominant role in activating the NF- κ B pathway. These results suggest that IKK α likely has targets other than the I κ B proteins. In the present study we show that the IKK complex can also regulate β -catenin protein levels and signaling activity. This is the first demonstration of an IKK substrate other than the I κ B proteins.

In contrast to the dominant role of IKK β in NF κ B signaling, our experiments show that IKK α is the dominant IKK component involved in β -catenin regulation. When over-expressed, both constitutively active IKK α and IKK β can decrease β -catenin signaling in SW480 cells, which have high endogenous levels of β -catenin. However, these kinases have no detectable effect in SKBR3 cells, which have low endogenous levels of β -catenin. Only the dominant negative IKK α , but not IKK β , can increase β -catenin protein levels and signaling in SKBR3 cells. Furthermore, only the dominant negative IKK α , but not IKK β , can reverse the inhibitory effects of APC on β -catenin signaling in SW480 cells. Taken together these data suggest a model in which signals that activate the IKK complex through IKK α can target a complex containing β -catenin whereas signals that activate the IKK complex through IKK β target it for I κ B α . Alternatively, it is possible that a subcomplex of IKK α homodimers can bind to β -catenin. A role for IKK α in the regulation of β -catenin signaling in epithelial cells is consistent with its potential role in the described phenotype of the IKK α $-/-$ mouse and of epidermal cells over-expressing β -catenin. Each of these phenotypes is characterized by hyperproliferation of basal stem cells (14;21;40;45). A role for the IKK α / β -catenin/TCF pathway in the regulation of epithelial stem cell proliferation is also consistent with the phenotype of the TCF4 $-/-$ mouse which completely lacks the stem cell compartment of the intestine (9).

The precise mechanism by which IKK regulates β -catenin is not clear but is likely to involve phosphorylation of one or more of the N-terminal serine and threonine residues known to regulate the stability and perhaps nuclear localization of both proteins (8;43). Like β -catenin, I κ B α has a function in both the cytoplasm and the nucleus (1). In addition to its role as a cytoplasmic inhibitor of NF- κ B nuclear translocation, nuclear I κ B α can also directly inhibit NF- κ B mediated transcriptional activation by preventing its DNA binding properties. Similarly, cytoplasmic β -catenin is involved in cell-cell adhesion and in the indirect regulation of PKA-regulated genes (?) in addition to its role as a nuclear co-activator of TCF/LEF regulated transcription (4;19). Remarkably, both I κ B α and β -catenin can also interact with and regulate the function of retinoid receptors. I κ B α can negatively regulate RXR-mediated transcription whereas β -catenin can act as a co-activator of RAR-mediated transcription. Both β -catenin and NF- κ B can bind to the coactivator protein CBP. Our results show that both IKK α and IKK β are present in the cytoplasm but that only IKK α is able to localize to the nucleus. This differential localization of the two IKK kinases may be relevant for the precise regulation of both nuclear and cytoplasmic functions of I κ B α and β -catenin. For example it is possible that IKK α could have two roles in the regulation of β -catenin

signaling. IKK α could phosphorylate β -catenin and target it for ubiquitination and could also directly interact with it to prevent its co-activator function in the nucleus. Perhaps IKK β can also regulate β -catenin phosphorylation in the cytoplasm but lacks the nuclear activity of IKK α .

Although APC is most commonly associated with wnt signaling, genetic studies differ on its role in this pathway including whether it antagonizes or potentiates wnt signaling (36). Similarly, although genetic studies show that GSK-3 β is a component of the wnt pathway and that inhibition of GSK-3 β activity elevates β -catenin signaling and protein levels in several different in vitro systems, GSK-3 β activity is not required for APC to regulate β -catenin signaling or protein levels (7). However the present study shows that IKK α (but not IKK β) is required for APC-mediated inhibition of β -catenin signaling. Other data shows that the phosphorylation event that results in the formation of the slower migrating form of β -catenin only occurs when β -catenin is overexpressed in cells expressing wild-type APC (Fig. 1D). The generation of this slower migrating species is completely blocked by co-expression of DN IKK α . These data indicate that elevation of β -catenin protein levels, by wnt signaling for example, results in the increased activity of a kinase (IKK α ?), which in turn phosphorylates the accumulated cytoplasmic and nuclear β -catenin to target it for degradation (Figure 6). In this model the IKK complex could be involved in the feedback regulation of pathways, such as wnt signaling, that result in elevated β -catenin levels. IKK α protein levels are similar in cells that express very low levels of β -catenin (SKBR3 cells) and in cells that express extremely high levels of β -catenin (SW480) and indicates that β -catenin does not regulate IKK α protein levels. Consequently, we favor a model in which elevated β -catenin protein levels directly trigger activation of the IKK complex. Perhaps the role of APC is to facilitate the β -catenin-mediated activation of the IKK complex and complete the negative feedback loop. Our demonstration that constitutively active IKK can decrease β -catenin signaling independently of APC is consistent with this model. β -catenin can also regulate the levels of the ubiquitin ligase component β TRCP which can in turn increase I κ B α degradation and subsequent NF κ B activation as well as negatively regulating its own activity (39). Taken together with the recent demonstration that GSK-3 is involved in the regulation of NF κ B activity, these data indicate that there is a significant amount of "cross-talk" between these two pathways (3;13). Our results strongly suggest that substrate specificity of the IKK complex is regulated by the relative contribution of IKK α and IKK β . Determining the precise mechanism by which IKK regulates β -catenin signaling as well as the factors specifying activity will further elucidate the relationship between these two important pathways.

Figure Legends

Figure 1: IKK α regulates β -catenin signaling and protein levels.

A luciferase reporter containing Tcf/LEF response elements (TopFlash) was used to detect the effects of IKK on β -catenin signaling. All transfections were normalized with equal amounts of DNA and performed in triplicate. Data is measured in lumens and plotted as percent control. A) SW480 cells were transfected using lipofectamine plus with PCDNA3-cat control DNA and/or 0.1 μ g constitutively active (CA) IKK α (S176/180E) or IKK β (S177/81) as well as 0.1 μ g of TopFlash and 0.002 μ g of Renilla luciferase, which was transfected as a control for transfection efficiency. Both CA IKK α and CA IKK β decreased β -catenin signaling by 80-90%. B) SW480 cells were transfected with 0.2 μ g of kinase dead IKK α (K44M) or IKK β (K44A), 0.1 μ g of TopFlash, and 0.002 μ g of Renilla. Kinase dead IKK α and β had no effect on β -catenin signaling indicating that IKK kinase activity is necessary. C) SKBR3 cells were transfected with 1 μ g of dominant negative (DN) IKK α (176/190A) or IKK β (S177/181A) as well as 1 μ g of TopFlash, and 0.02 μ g Renilla. DN IKK α increased β -catenin signaling by over 5 fold whereas DN IKK β had no effect. TopFlash, which is a Tcf/LEF mutated luciferase reporter, was used as a control and was unaffected by IKK. D) SKBR3 cells were transfected with 10 μ g DN IKK α or β . NP-40 lysates were probed using a monoclonal antibody to β -catenin. DN IKK α significantly increased β -catenin protein levels whereas DN IKK β had no effect. SKBR3 cells were also co-transfected with 10 μ g of wild-type (WT) β -catenin and DN IKK α and probed for β -catenin expression. Overexpression of WT β -catenin generates a phosphorylated form of β -catenin and two bands (arrows). Co-expression of DN IKK α prevents this phosphorylated form of β -catenin. E) NP-40 lysates of cell lines used in this study were probed using monoclonal antibodies to IKK α and β . These cells have significantly more IKK α than IKK β indicating that IKK α has a more prominent role. Jurkat cell lysates were used as a control.

Figure 2: DN IKK α increases nuclear β -catenin levels.

Immunocytochemistry was performed to determine the effect of DN IKK α on β -catenin localization in SKBR3 cells. Cells were transfected with 1 μ g of HA-tagged DN IKK α for 48 hours, fixed in 2% paraformaldehyde and 0.2% Triton, and stained with monoclonal HA antibody (A1). DN IKK α was found in both the cytoplasm and nucleus in the majority of transfected cells. These cells were also double-stained for endogenous β -catenin using a polyclonal β -catenin antibody (A2). β -catenin levels were significantly higher in transfected cells and in most cases co-localized with DN IKK α in the nucleus. In SKBR3 cells transfected with 1 μ g of FLAG-tagged DN IKK β , DN IKK β was found predominantly in the cytoplasm (B1). Increased β -catenin protein levels were not observed nor was β -catenin detected in the nucleus.

Figure 3: IKK α regulates cyclin D1 promoter activity.

To determine if IKK could regulate the expression of a β -catenin target gene, a cyclin D1-luciferase promoter (0.5 μ g) was used. Both CA IKK α and CA IKK β decreased cyclin D1 promoter activity in SW480 cells. (Note that data is reported as arbitrary units.) A cyclin D1 promoter with mutated Tcf/LEF sites (Δ LEF) was used as a control.

Figure 4: DN IKK α inhibits the ability of APC and APC2 to decrease β -catenin signaling.

To determine the effects of IKK α on APC activity, SW480 cells were co-transfected with 0.1 μ g APC and 0.1 μ g DN IKK α or β in addition to TopFlash and Renilla. APC decreases β -catenin TopFlash

signaling by 80 %. (Data is reported as percent control.) DN IKK α but not DN IKK β completely inhibited the ability of APC to decrease β -catenin signaling.

Figure 5: IKK α interacts with β -catenin.

Superdex-200 column fractions derived from S100 Cos cells trasfected with Flag-tagged IKK α and Ha-tagged β -catenin were immunoprecipitated with mouse IgG (lane 1 and 3) or anti-Flag (lane 2) or anti-HA antibody (lane 4). The immunoprecipitates were subjected to fractionation by SDS PAGE, and immunoblotted with antibodies to HA (lanes 1 and 2) or Flag (lanes 3 and 4). β -catenin associates with IKK α .

Figure 6: Potential model for IKK α regulation of β -catenin.

Wnt signaling increases β -catenin protein levels by inactivating GSK-3. We have shown that IKK α directly regulates β -catenin protein levels and signaling activity. This suggests that elevation of β -catenin leads to IKK α activation and subsequent β -catenin phosphorylation and degradation creating a feedback loop. It is possible that β -catenin regulates transcription of an IKK kinase, such as NIK, thus activating IKK and down-regulating itself. We also showed that IKK α activation is necessary for APC regulated inhibition of β -catenin signaling. APC may be necessary to facilitate β -catenin targeting by IKK α . In addition, β TRCP is upregulated by β -catenin, consequently decreasing β -catenin levels and down-regulating its own activity as well as increasing NF- κ B activity. These data indicate a significant amount of "cross-talk" between these two pathways.

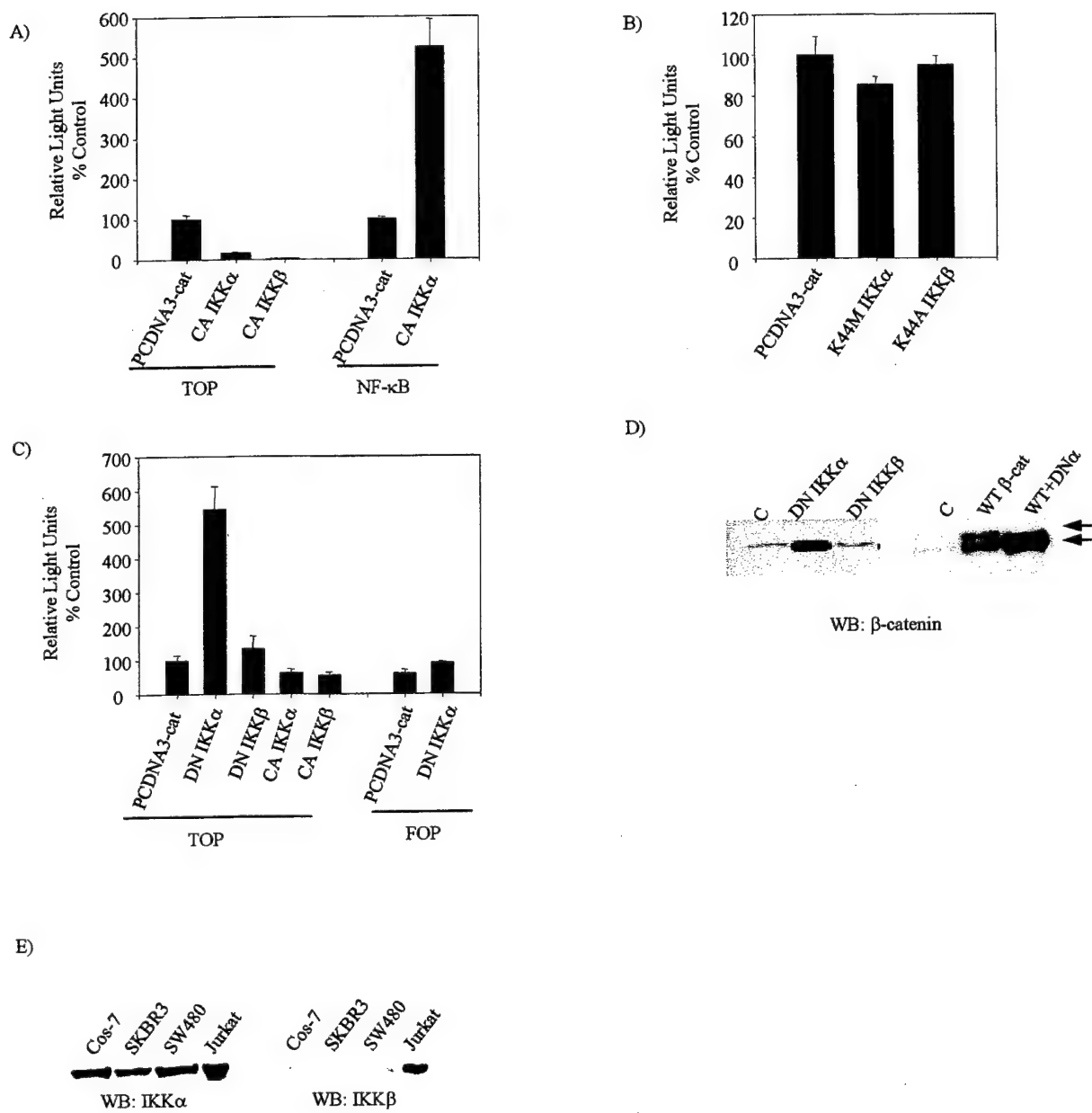


Figure 1

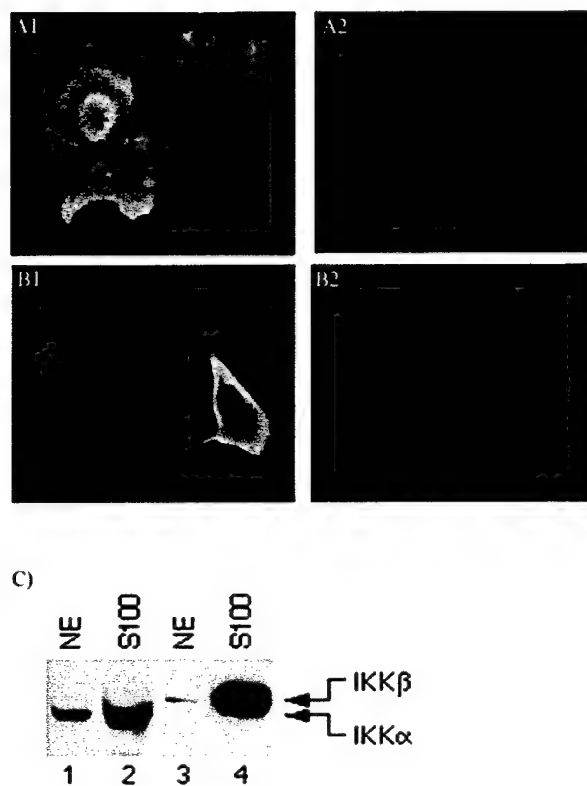


Figure 2

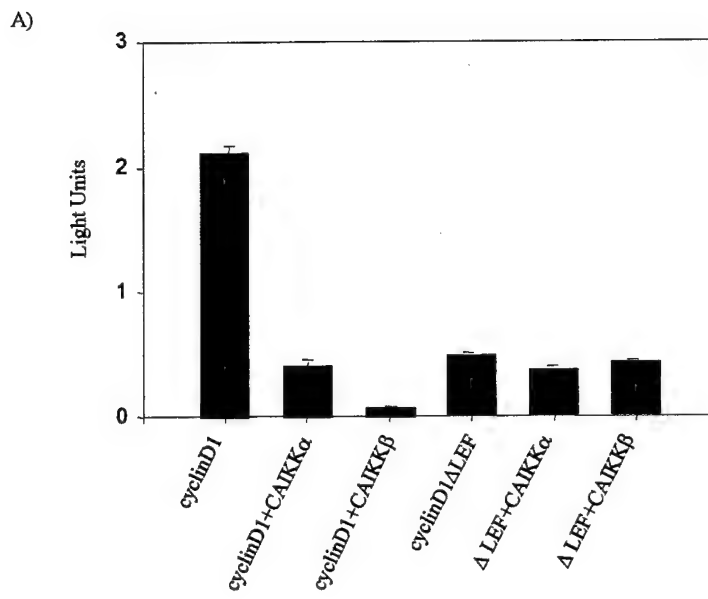


Figure 3

A)

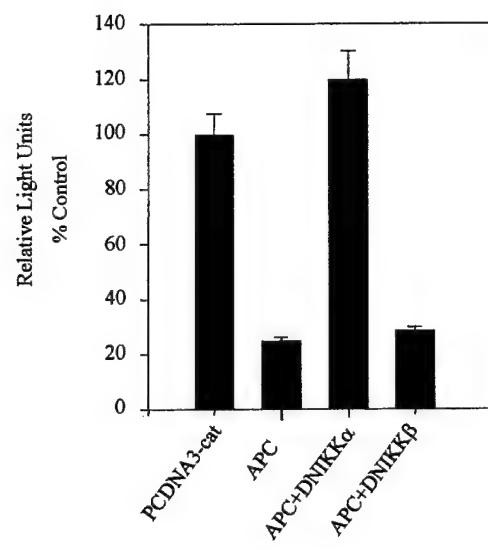


Figure 4

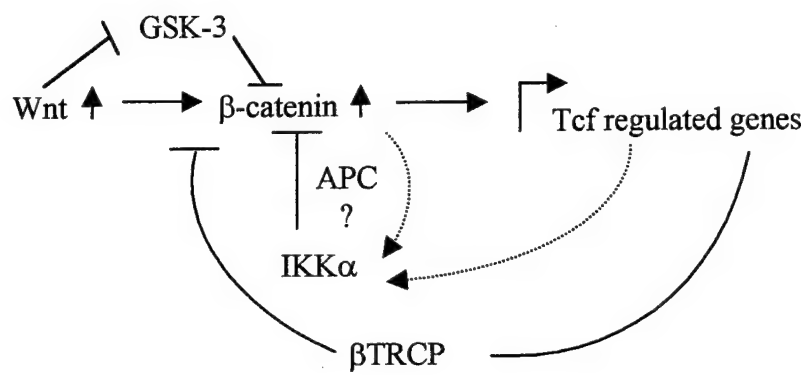


Figure 6

Key Research Accomplishments:

- 1) Identification of the IKK complex as a β -catenin-directed kinase
- 2) Demonstration that IKK α , not IKK β is the active kinase
- 3) Demonstration that the activity of the tumor suppressor APC requires IKK α , but not IKK β

Reportable Outcomes: The IKK complex regulates β -catenin signaling. Paper in preparation

Conclusions: The wnt/ β -catenin and NF κ B pathways regulate the transcription of genes that are involved in cell cycle control and cellular differentiation. In addition the NF- κ B pathway is involved in the induction of the inflammatory response. β -catenin, a known oncogene, is an important component of the wnt signaling pathway and I κ B α is an important regulator of the NF- κ B pathway. Both proteins are phosphorylated at serines in the N-terminal region, which subsequently target them for ubiquitination by the same ubiquitin ligase complex. The kinases that are important in the phosphorylation of these proteins have been intensely studied. The IKK complex is responsible for the phosphorylation of I κ B α while GSK-3 β is thought to regulate β -catenin phosphorylation. The IKK complex contains two kinases, IKK α and IKK β . Gene disruption studies in mice indicate that IKK β is the dominant component of the IKK complex involved in phosphorylation of I κ B α . We now show that IKK also exists in a complex with β -catenin and that expression of either IKK α or IKK β can decrease β -catenin signaling in APC-mutant colon cancer cells with high endogenous β -catenin levels. However, only a dominant negative (DN) IKK α mutant increased β -catenin signaling and protein levels in cells with low endogenous β -catenin. In addition, DN IKK α , but not DN IKK β , completely inhibited the ability of APC to decrease β -catenin signaling in colon cancer cells. These results indicate that, in contrast to IKK control of NF κ B signaling, IKK α not IKK β has the dominant role in the regulation of β -catenin activity.

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